



Europäisches Patentamt
European Patent Office
Office européen des brevets



Publication number: **0 302 284 B1**

(12)

EUROPEAN PATENT SPECIFICATION

(43) Date of publication of patent specification: 30.09.92 (51) Int. Cl.⁵: **C12N 9/96, C12N 11/08**

(21) Application number: **88111443.3**

(22) Date of filing: **15.07.88**

(54) **Stabilized enzymes.**

(30) Priority: **06.08.87 IL 83451**

(43) Date of publication of application:
08.02.89 Bulletin 89/06

(45) Publication of the grant of the patent:
30.09.92 Bulletin 92/40

(84) Designated Contracting States:
AT BE CH DE ES FR GB GR IT LI LU NL SE

(56) References cited:
EP-A- 0 049 475
GB-A- 2 146 029

BIOTECHNOLOGY AND BIOENGINEERING,
Band 23, Nr. 12, Dezember 1981, Seiten
2747-2759, John Wiley & Sons, INC., New
York, US; A. FREEMAN: "Immobilization of
microbial cells in crosslinked, prepolymerized,
linear polyacrylamide gels: antibiotic
production by immobilized streptomyces
clavuligerus cells"

CHEMICAL ABSTRACTS, Band 76, Nr. 9, 28.
Februar 1972, Seite 167, Nr. 43458s, Colum-
bus, Ohio, US; P.D. WESTON et al.: "Proteins
coupled to polyacrylamide beads using
glutaraldehyde", & BIOCHEM. BIOPHYS. RES.

COMMUN. 1971, 45(6), 1574-80

(73) Proprietor: **RAMOT UNIVERSITY, AUTHORITY**
FOR APPLIED RESEARCH & INDUSTRIAL DE-
VELOPMENT LTD.
Tel Aviv University, 32, University Street, P.O.
Box 39296, Ramat Aviv
Tel Aviv 61392(IL)

(72) Inventor: **Freeman, Amihay**

Ben Shemen 71 904(IL)
Inventor: Tor, Ruth
Bialik Street 43
Kiryat Ono 55203(IL)

(74) Representative: **Brown, John David et al**
FORRESTER & BOEHMERT Franz-
Joseph-Strasse 38
W-8000 München 40(DE)

Note: Within nine months from the publication of the mention of the grant of the European patent, any person may give notice to the European Patent Office of opposition to the European patent granted. Notice of opposition shall be filed in a written reasoned statement. It shall not be deemed to have been filed until the opposition fee has been paid (Art. 99(1) European patent convention).

Description**FIELD OF INVENTION**

5 The invention concerns a stabilized enzyme product and a method for its preparation. The stabilized enzyme product may be in form of an aqueous solution or a lyophilized dry product. A stabilized enzyme produce according to the invention may optionally be further stabilized by immobilization.

LIST OF REFERENCES

- 10 1. Klivanov, A.M., *Adv. Appl. Microb.* 1983, 29, 1-28.
2. Martinek, K. and Berezin, I. V., *J. Solid Phase Biochem.* 1978, 2, 343-385.
3. Butler, L. G., *Enzyme Microb. Technol.*, 1979, 1, 253-259.
4. Martinek, K., Klivanov, A.M., Goldmacher, V.S. and Berezin, I.V., *Biochim. Biophys. Acta* 1977, 485, 1-12.
- 15 5. Klivanov, A.M., *Anal. Biochem.* 1979, 93, 1-25.
6. Schmid, R.D. *Adv. Biochem. Eng.* 1979, 12, 41-118.
7. Back, J.R., Oakenfull, D. and Smith, M.B., *Biochemistry* 1979, 18, 5191-5196.
8. Tonchilin, V.P. and Martinek, K., *Enzyme Microb. Technol.* 1979, 1, 74-82.
9. Greco, G. and Gianfreda, L., *Biotechnol. Bioeng.* 1981, 23, 2199-2210.
- 20 10. Wykes, J.R., Dunill, P. and Lilly, M.D., *Biochim. Biophys. Acta* 1971, 250, 522-529.
11. Marshall, J.J., *Trends in Biochem. Sci.* 1978, 3, 79-83.
12. Lenders, J.P. and Crichton, R.R., *Biotech. Bioeng.* 1984, 26, 1343-1351.
13. Hixon, H.F., *Biotech. Bioeng.* 1973, 15, 1011-1016.
- 25 14. von Specht, B.U., Seinfeld, H. and Brendel, W., *Hoppe-Seyler's Z. Physiol. Chem.* 1973, 354(s), 1659-1660.
15. Paz, M.A., Blumenfeld, O.O., Rojkind, M., Henson, E., Furfine, C., and Gallop, P.M., *Arch. Biochem Biophys.*, 1965, 109, 548-559.
16. Gary, W.L., Johnson, R.N. and Kho, B.T., *J. Chromatog.* 1978, 156, 285-291.
- 30 17. Freeman, A., *Methods. Enzymol.* 1987, 135, 216-222.
18. Collinson, E., Dainton, F.S. and McNaughton, G.S., *Trans. Faraday Soc.* 1957, 53, 489-498.
19. Freeman, A. and Aharonowitz, Y., *Biotech. Bioeng.*, 1981, 23, 2747-2759.
20. Reuveny, S., Mizrahi, A., Kotler, M. and Freeman, A., *Biotech. Bioeng.* 1983, 25, 469-480.
21. Blank-Koblenc, T., Tor, R. and Freeman, A., *Biotechnol. Appl. Biochem.*, 1988, 10, 32-41.
- 35 22. Nichols, C.S., and Cromartie, T.H., *J. Chem. Educ.*, 1979, 56, 832-834.
23. Samuni, A., *Anal. Biochem.*, 1975, 63, 17-26.
24. Malikkides, C.O. and Weiland, R.H., *Biotech. Bioeng.* 1982, 24, 1911-1914.
25. Freeman, A., Blank, T. and Haimovich, B., *Annal. N.Y. Acad. Sci.*, 1983, 413, 557-559.
26. Goldstein, L. and Manecke, G., *Appl. Biochem. Bioeng.*, 1976, 1, 23-126.
- 40 27. Dror, Y., Cohen, O. and Freeman, A., *Enz. Microb. Technol.*, 1988, 10, 273-279.

BACKGROUND OF THE INVENTION AND PRIOR ART

45 One of the intrinsic problems often hindering further development of analytical and synthetic applications of enzymes is the inherent instability of many enzymes. This limitation is crucial in particular in the case of applications involving prolonged exposure of the enzyme to elevated temperatures or to the presence of organic solvents (refs. 1, 2, 3).

Loss of enzymic activities may result from several factors imposing their effect via different mechanisms, of which the structural unfolding of the enzyme is the most abundant (refs. 2,4).

50 Enzyme stabilization by means of restrictions imposed on the enzyme, limiting its large conformational changes, was in the past attempted by either of two main approaches: rigidification of protein structure by multi-point attachment to water insoluble polymeric supports (refs. 1, 2, 4, 5), or by the addition of various solutes (refs. 6, 7, 8,9).

Several reports in literature describe coupling of water soluble polymers to enzymes, aiming towards 55 the stabilization of enzymes in soluble form (for review see ref. 1). Thus, water soluble enzyme-polymer conjugates, prepared by enzyme coupling to polysaccharides (refs. 1, 10, 11, 12), polyvinylalcohol (ref. 13), polyvinylpyrrolidone (ref. 14) and polymethacrylic acid (ref. 4) were described.

Effective stabilization of soluble enzymes via this approach strongly depends on obtaining an intensive

5 multipoint polymer - enzyme interaction, in a complementary way (ref. 1,4). The effectiveness of such enzyme - polymer conjugates will also depend on the chemical nature of the polymeric moiety, creating a hydrophylic or charged microenvironment in the immediate surrounding of the enzyme. The most effective conjugate of this kind seems to be the chymotrypsin-polymethacrylic acid conjugate, prepared by the
 10 copolymerization of methacrylic acid and acryloyl chloride treated enzyme (ref. 4). The resulting poly - anionic enzyme derivative exhibited stability comparable to that of the gel entrapped enzyme analogue, prepared by copolymerization of methacrylamide or acrylamide, with bisacrylamide and acryloyl chloride treated enzyme.

10 SUMMARY OF THE INVENTION

In accordance with the invention there is provided a stabilized, biologically active water soluble enzyme product, characterized by enzyme molecules having a bi-layer protective structure comprising a polyaldehyde base coat linked to free amino groups of the enzyme, and cross-linked therewith an outer polymer
 15 coat, the polymers constituting said outer coat being of a kind which in the unlinked state comprises free amino and/or acyl hydrazide groups.

The invention further provides a method of making a stabilized, biologically active enzyme product of the kind specified, characterized in that in a first step a native enzyme product is reacted in aqueous solution with an excess of a water soluble polyaldehyde to produce an intermediary enzyme product in
 20 which the enzyme molecules are coated with said polyaldehyde base coat, and in a second step said intermediary enzyme product is reacted in aqueous solution with a polymeric reagent bearing free amino and/or free acyl hydrazide groups.

The bi-layer protective structure provided in accordance with the invention will occasionally be referred to hereinafter as "cage" and its production as "encagement". It provides several advantages over the prior
 25 act, such as:

- The "cage" concept is of a general nature and enables also the stabilization of enzymes bearing low numbers of lysil residues. This is so because the polyaldehyde used in the first step amplifies the total number of crosslinking bridges formed and consequently, as distinct from the prior art (ref. 4) the stabilization degree will not depend solely on the lysil content of the enzyme (ref. 4).
- 30 - The encagement method is based on the use of water soluble polymeric reagents, and is thus virtually free of toxic and inhibitory effects which occur when low molecular weight reagents have to be used such as, for example, acryloyl chloride, and acrylamide.
- The "cage" concept allows for control of the microenvironment of the "encaged" enzyme by suitably modifying or selecting the chemical nature of the polymeric reagent used in the formation of the outer coat. For example, hydrophylicity or hydrophobicity may be introduced into the said polymeric
 35 reagent as may be required by the nature of the enzyme or its intended use.
- Due to the combined effect of the two constituent "cage" coats, the stabilization of the enzyme is more effective as compared to single coat enzyme-polymer conjugates.

The nature of the reactants in said first and second steps is not critical. Thus, in the first step any water
 40 soluble polyaldehyde may be used that is capable of reacting with free amino groups forming part of lysil residues of the enzyme, a typical example being polyglutaraldehyde. Likewise there is no criticality in the selection of the water soluble polymeric reagent used in the second step, provided it bears free amino and/or acyl hydrazide groups, since it is these groups that cross-link with carbonyl groups of the polyaldehyde base coat. Typical examples of polymeric reactants used in the second stage are
 45 polyacrylamides, N-alkylated polyacrylamides, acylhydrazide or amino derivatives of polyacrylamides, polyvinylhydrazines, polyvinylamines, polylysine, various proteins and the like.

If desired, a stabilized enzyme product with "encaged" enzyme molecules according to the invention may be further stabilized by immobilization. As the outer coat of the stabilized enzyme according to the invention is made of polyacrylamide-amine or acyl hydrazide derivatives, its immobilization, either by
 50 chemical binding or crosslinking, is readily available. The latter method is particularly suitable seeing that the stabilized enzyme solution obtained at the end of the second step contains a large excess of unbound polymer which may be employed to form a matrix by appropriate crosslinking. Immobilization of enzymes by means of gel matrices is known per se and was successfully employed for the immobilization of enzymes in gels made of polyacrylamide-hydrazide crosslinked by glyoxal (refs. 21, 25).

55 It is believed that the stabilization of "encaged" enzymes according to the invention results from a combination of two effects. For one there occurs physical suppression of the unfolding process by the crosslinked polymeric "cage" built around the enzyme. Secondly there occurs a chemical suppression of conformational changes due to the microenvironmental effect of the bound hydrophylic polymeric moiety.

A stabilized enzyme product according to the invention may be stored in aqueous solution or be lyophilized into dry powder and stored as such. It has surprisingly been found in accordance with the invention that lyophilization of the "encaged" enzyme product according to the invention further increases its stability. Furthermore, immobilization of lyophilized "encaged" enzyme in glyoxal crosslinked polyacrylamide-hydrazide gel results in maximal amplification of this stabilization approach.

The stabilized and optionally immobilized enzyme products according to this invention may be used in a variety of enzyme catalyzed chemical processes, whether carried out batchwise or continuously.

DESCRIPTION OF THE DRAWINGS

10

For better understanding the invention will now be described with reference to the annexed drawings, in which:

Fig. 1 is a diagram showing the stabilization of pig liver esterase by "encagement" in accordance with the invention expressed by resistance to combined temperature -cosolvent denaturation effects by three different solvents, in comparison to the behaviour of native carboxylesterase under similar conditions;

Fig. 2 is a diagram showing the thermal stability of a lyophilized "encaged" pig liver esterase entrapped in a polyacrylamide-hydrazide gel in accordance with the invention, in comparison to the behaviour of native pig liver esterase under similar conditions; and

Fig. 3 is a schematic illustration of the formation a bi-layer protective cage in accordance with the invention.

20

DESCRIPTION OF THE INVENTION

The stabilization of "encaged" enzymes according to the invention in aqueous solution and in the presence of water miscible organic solvents was investigated. Stabilization against denaturation by organic co-solvents is of great practical value as such cosolvents may be employed for the enhancement of substrate and product solubilities and the reversal of "natural" reaction routes (refs. 3,21). The stabilization effect was estimated by measuring inactivation-rate constant (k_{in}) values for native and "encaged" enzymes (lyophilized) incubated at 55°C in the presence of 3.5M cosolvent (about 20% v/v; (ref. 21)). Cosolvents, representing the group exhibiting only mild interference with the retention of enzyme stability in their presence (e.g. ethylene glycol, DMSO (ref. 21)), as well as cosolvents exhibiting strong denaturing effect (e.g. ethanol) were employed and the stabilization according to the invention was found to be effective with solvents of both groups. In Fig. 1 the empty circles stand for a stabilized encaged carboxylesterase according to the invention and the blackened circles stand for the native enzyme, and it is clearly seen that the rate of inactivation of the former is much lower than of the latter. This enhanced stability against denaturation by ethylene glycol, DMSO and ethanol allows for a significant increase in the content of the cosolvent present, without impairing the enzymic activity. Stabilized enzyme products according to the invention can thus be used to advantage as catalysts in the performance of reactions in which co-solvents are required from the outset or form in the course of the reaction.

Fig. 2 shows that a lyophilized "encaged" and immobilized enzyme product according to this invention (empty circles) retained its activity over sixteen days virtually without any change, while that of a native enzyme (blackened circles) dropped sharply during day 1 and from then on approached asymptotically zero activity. In these tests the enzymes were incubated at 55°C in 0.05M Tris buffer (pH 8) and the activities were periodically tested.

Fig. 3 illustrates schematically the formation of a bi-layer "encagement" in accordance with the invention. As shown, in a first step an enzyme molecule is reacted with a polyaldehyde to yield an enzyme with a base coat. This product is then reacted with a "Polymer 2" being an amino and/or acyl hydrazide groups bearing water soluble polymer, to yield an enzyme within a bi-layered cage in accordance with the invention.

The invention will now be further described in the following examples to which it is not limited. All temperature indications are in centigrades.

Example 1

Stabilization of glucose oxidase (E.C.1.1.3.4) by bilayer encagement

1. Preparation of polymeric reagents:

(a) Water soluble polymeric glutardialdehyd :

Into a 100 ml round bottomed flask, thermostated at 50 ° C, 20 ml of 1M K₂CO₃ (pH = 10) were added, followed by 20 ml of 25% (w/v) glutardialdehyde (Merck, Cat. No. 4239). The polymerization reaction was allowed to proceed at 50 ° C for 2 h. The reaction mixture was then cooled to room temperature, the pH brought to 7 by the addition of concentrated HCl and precipitates removed by centrifugation (7000 rpm for 10 min.). The remaining solution was then added into a ten fold volume of acetone to remove K₂CO₃ and salts by precipitation. The solution was then separated, the acetone evaporated and the remaining solution lyophilized and stored at -20 ° C (yield: 3.3 g; 66%).

The water soluble polymeric glutaraldehyde thus obtained contained 0.35 mmole aldehyde groups per g (dry weight) determined according to J.S. Thompson and G.D. Shockman (1968) Analytical Biochemistry 22, 260-268) and its MW estimated from gel filtration ("Biogel" p-6) against protein standards to be 1000.

(b) Preparation of Polyacrylamides:

b-1) Polyacrylamide of MW = 8000:

Into a one liter round bottomed flask, equipped with a magnetic stirrer, 680 ml of water were added. The temperature was brought to 4 ° C by means of an ice bath and, under nitrogen flushing, 4 g (0.056 mole) of acrylamide monomer were added. Following complete monomer dissolution, 4.6 ml (0.034 mole) of N,N,N,N-tetramethylethylenediamine were added, followed immediately by 20 ml of 0.8777 M (4g in 20 ml) ammoniumpersulfate. The polymerization reaction (over ice, under nitrogen atmosphere) was allowed to proceed for one hour.

The polymer solution was then added dropwise into 3.5 l ice cold methanol, the precipitate separated by filtration, stored overnight at 4 ° C under methanol, separated and dried on a rotavapour for 30 min at 40 ° C. The polymer was finally dried by dessication in vacuo over P₂O₅. The dry polymer was stored in a tightly closed vessel at room temperature (yield 3.2 g (80%)).

b-2) Alkylamine - derivative:

Into a 0.25 l round bottomed flask equiped with a magnetic stirrer 100 ml ethyleneglycol were added and the temperature adjusted to 50 ° C (oil bath). One g of polyacrylamide was added and dissolution was allowed to proceed at 50 ° overnight. The temperature was then adjusted to 100 ° C and 13 ml (0.13 mole) 1,4 diaminobutane were added. The aminolysis reaction was allowed to proceed at 100 ° C for 3 hrs. The solution was then cooled over ice and mixed with 100 ml of ice cold 2N HCl. The pH of this solution was adjusted to 6.3 and the solution dialyzed (four times: against 5 litres of 0.02M phosphate, pH 6.3; 0.02M phosphate, pH 6.3; 0.02M phosphate, pH 6.3; and finally water). The polymer was finally recovered by lyophilization (yield: essentially quantitative, amine content 2 meq/g dry polymer (12.5% conversion, determined titrimetrically according to J.F. Inman and H.M. Dintzis, (1969) Biochemistry 8, 4074-4082) and stored at -20 ° C.

2. Bilayer encagement of glucose oxidase

2-a) Into a 20 ml beaker equipped with a magnetic stirrer and thermostated at 4 ° C, 9.8 ml of 10 mg/ml solution of polymeric glutaraldehyde in 0.2 M phosphate pH 8.2 were added, followed by 0.2 ml of 50 mg/ml glucose oxidase solution in 0.2 M phosphate pH 6.0. The coupling reaction was allowed to proceed for 3 hrs at 4 ° C. The non-bound polymer was removed by dialysis (3 times against 0.2M phosphate buffer pH 8).

2-b) Into a 20 ml beaker equipped with a magnetic stirrer and thermostated at 4 ° C, 16 ml of aminobutyl derivative of polyacrylamide solution (0.25 mg/ml in 0.2M phosphate, pH 8.0) were added, followed by 4 ml of polyglutaraldehyde coated glucose oxidase solution (obtained as described above). The reaction was allowed to proceed for 3 hrs at 4 ° C and the pH adjusted to 6.0 by dialysis against 0.2M phosphate pH 6.0.

The stabilized enzyme was stored either as solution at 4 ° C or as a lyophilized powder.

3. Stabilization of glucose oxidase to thermal inactivation:

The stabilization of glucose oxidase to thermal denaturation was demonstrated by measuring the rate of inactivation of the native or stabilized enzyme at a fixed elevated temperature (55 ° C). The rate constant

defined as:

$$K_{\text{ina}} = - \frac{\ln E(t)/E(o)}{t}$$

where: $E(t)$ = enzymic activity measured at time "t"

E(o) = " " " " " "o"

(for description of this method see ref. 24).

The data are presented in Table 1.

Table 1

Effect of bi-layer encagement on the thermal stability of glucose oxidase			
Enzyme	K _{ina} (h ⁻¹ , 55 °C)	Relative K _{ina} (%)	Residual activity after 5 h at 55 °C (%)
native (control)	0.19	100	43
polyglutaraldehyde-treated	0.066	35	60
bi-layer ("encaged")	0.032	17	80

4. Stabilization of glucose oxidase to inactivation resulting from the presence of organic cosolvents

The stabilization of glucose oxidase towards the denaturing effect of water miscible organic solvents was demonstrated by measuring the rate of inactivation (K_{in}) for native and stabilized enzyme at a fixed elevated temperature (55 °C) in presence of 3.5 M (20%) of cosolvent. The data are presented in table 2.

Table 2

Effect of bi-layer encagement on the tolerance of glucose oxidase to organic solvents.			
Co-solvent (all at 3.5M)	K _{ina} (h ⁻¹ ·55 ° C)		Relative (b/a,%)
	Native enzyme (a)	Encaged enzyme (b)	
none	0.19	0.055	29
ethyleneglycol	0.16	0.074	46
DMSO	0.19	0.075	40
DMF	0.40	0.22	55
ethanol	1.020	0.40	39
formamide	1.74	0.50	29

Example 2

Stabilization of pig liver esterase (E.C.3.1.1.1.) by bi-layer encagement.

1. preparation of polymeric reagents

Polymeric polyglutaraldehyde and polyacrylamide were prepared as described in Example number 1.

An acylhydrazide-derivative of polyacrylamide was prepared as follows: Into a 100 ml round bottomed flask equipped with a magnetic stirrer and maintained at 50° C, 38 ml of water and 1.5g of polyacrylamid were added. Following complete dissolution 12 ml (0.23 mole) hydrazine hydrate wer added and the

hydrazinolysis reaction allowed to proceed for 4 hrs. The acylhydrazide derivative thus obtained was separated by precipitation induced by the dropwise addition of the aqueous reaction solution into 250 ml of methanol, recovered by centrifugation, redissolved and reprecipitated as above, stored overnight at 4 °C under methanol, separated and dried on a rotavapour (30 min at 40 °C) and finally dessicated in vacuo over P_2O_5 . The dry polymer (acyl-hydrazide content: 1.5 mmole/gm, determined according to ref. 19, yield: essentially quantitative) was stored at -20 °C.

2. Bilayer encagement of pig-liver esterase

2-a) Into a 50 ml beaker equipped with a magnetic stirrer and thermostated at 4 °C, 19.7 ml of 4 mg/ml polygluteraldehyde solution in 0.05 M phosphate pH 8.0 were added followed by 0.3 ml of 11 mg/ml enzyme stock solution (Sigma, Cat. No. E-3128). The coupling reaction was allowed to proceed at 4 °C for 3 hrs and excess of polygluteraldehyde was removed by dialysis (three times against 0.05 m phosphate, pH 8.0).

2-b) Into a 100 ml beaker, equipped with a magnetic stirrer and thermostated at 4 °C, 41 ml of 5 mg/ml acylhydrazide derivataive of polyacrylamide in 0.05M phosphate, pH 8, were added, followed by 19 ml of polygluteraldehyde enzyme solution (obtained as described above). The reaction was allowed to proceed at 4 °C for 3 hrs and finally dialysed against 0.05 m Tris buffer pH 8. The stabilized enzyme was stored as solution at 4 °C or as lyophilized powder.

3. Stabilization of pig liver esterase to thermal inactivation

The stabilization of pig liver esterase to thermal denaturation was demonstrated by measuring Kina ($h^{-1}, 55^{\circ}C$) as described in Example 1. The data are presented in Table 3.

Table 3

Effect of bilayer encagement on the thermal stability of pig liver esterase.			
Enzyme	Kina ($h^{-1}, 55^{\circ}C$)	Relative Kina (%)	Residual activity after 5 h at 55 °C (%)
native (control)	0.140	100	47
polygluteraldehyde-treated	0.047	34	50
bi-layer ("encaged")	0.032	23	82

4. Stabilization of pig liver esterase to the presence of water miscible solvents

Stabilization to the denaturing effect of water miscible cosolvents present in 3.5 M ($\approx 20\%$) concentration was demonstrated as described in Example 1. The data are presented in Table 4 and they relate to lyophilized encaged enzyme preparations.

Table 4

Effect of bilayer encagement on the tolerance of pig liver esterase to organic solvents.		
Co-solvent (all at 3.5M)	Kina($h^{-1}, 55^{\circ}C$)	
	Native enzyme	Encage enzyme (lyophilized)
none	0.14	0.00
ethyleneglycol	0.24	0.00
DMSO	0.24	0.01
propyleneglycol	0.50	0.00
ethanol	2.95	0.093

Example 3

Stabilization of β -lactamase (E.C.3.5.2.6) bilayer encagement

1. Preparation of polymeric reagents: polyglutaraldehyde was prepared as described in Example 1. Polyacrylamide, partially substituted with acylhydrazide groups was prepared as described in Example 2.
2. The two stage procedure was carried out essentially as described in Example 2 for pig liver esterase.
3. Stabilization of β -lactamase to thermal denaturation by bilayer encagement according to the invention was demonstrated by measuring K_{ina} (h^{-1} , 55°C) as described in Example 1. The data are shown in Table 5.

Table 5

Effect of bi-layer encagement on the thermal stability of β -lactamase			
Enzyme	K_{ina} (h^{-1} , 55°C)	Relative K_{ina} (%)	Residual activity after 5 h at 55°C (%)
native (control)	0.52	100	10
polyglutaraldehyde treated	0.10	19	28
bi-layer ("encaged")	0.03	6	70

Example 4Stabilization of "caged" enzymes by lyophilization

Lyophilization of "caged" enzymes resulted in significant further improvement of enzyme stability. This effect is in contrast to the common effect of lyophilization on native enzymes; in many cases their stability declines, in others it remains unchanged.

The effect of lyophilization on "caged" enzyme stability is demonstrated in Table 6 for pig liver esterase and β -lactamase.

Table 6: Effect of lyophilization on the thermal stability of "caged" and native enzymes

Enzyme	$K_{ina}(h^{-1}, 55^{\circ}C)$	
	Before lyophilization	Following lyophilization
Pig-liver esterase-native	0.12	0.14
" " " - "caged"	0.032	0.00
β lactamase - native	0.52	2.59
" " - "caged"	0.03	0.00

Example 5: Immobilization and stabilization of lyophilized "caged" enzymeImmobilization procedur

Into a 1.5 g solution of polyacrylhydrazide (MW 100,000) in 8.5 ml of distilled water, 0.5 ml of 1M phosphate buffer (pH 8) was added and thoroughly mixed. A buffered solution of a lyophilized "encaged" pig liver esterase prepared as in Example 2 (150 mg in 1 ml of 0.05M phosphate, pH 8, 30 EU) was then added, thoroughly mixed by magnetic stirring and the solution thus obtained injected (injector outlet 2 mm) into an ice-cold 1% glyoxal solution. The gel "noodles" thus obtained were allowed to harden for one hour and then fragmented into fragments of about 2 mm by pressing through a syringe (outlet diameter 2mm). The gel was then incubated at 4°C for 20h and further fragmented into particles of about 0.25 mm by means of a blade homogenizer ("Sorval Omnimixer", 7000 rpm, 1 min). The gel particles were washed with 1 litre of ice cold 0.05M Tris buffer (pH 8), resuspended in 50 ml of the same buffer and stored at 4°C until used.

The thermal stability - at 55°C - of this prepare is shown in Fig. 2.

The features disclosed in the foregoing description, in the claims and/or in the accompanying drawings may, both separately and in any combination thereof, be material for realising the invention in diverse forms thereof.

Claims

1. A stabilized, biologically active water soluble enzyme product, characterized by enzyme molecules having a bi-layer protective structure comprising a polyaldehyde base coat linked to free amino groups of the enzyme, and cross-linked therewith with an outer polymer coat, the polymers constituting said outer coat being of a kind which in the unlinked state comprises free amino and/or acyl hydrazide groups.
2. A method of making a stabilized, biologically active enzyme product according to claim 1, characterized in that in a first step a native enzyme product is reacted in aqueous solution with an excess of a water soluble polyaldehyde to produce an intermediary enzyme product in which the enzyme molecules are coated with said polyaldehyde base coat, and in a second step said intermediary enzyme product is reacted in aqueous solution with a polymeric reagent bearing free amino and/or free acyl hydrazide groups.
3. A method according to claim 2, characterized in that the product obtained in said second step is lyophilized.
4. An enzyme product according to claim 1, characterized by being immobilized within a matrix.
5. A method of making an immobilized enzyme product according to claim 4, characterized in that an aqueous solution obtained at the end of said second step as defined in claim 2 and which contains unreacted polymer, is subjected to a cross-linking reaction.
6. An enzyme product according to claim 1 characterized by being in form of an aqueous solution.
7. An enzyme product according to claim 1, characterized by being a lyophilized dry product.
8. An enzyme product according to any one of claims 1, 4, 6 and 7, characterized in that said base coat is made of polyglutaraldehyde.
9. An enzyme product according to any one of claims 1, 4, 6, 7 and 8, characterized in that said outer coat is made of a polymer selected from the group of polyacrylamides, N-alkylated polyacrylamides and acylhydrazide derivatives of polyacrylamides.
10. An enzyme product according to any one of claims 1, 4, 6, 7, 8 and 9, characterized in that the enzyme is glucose oxidase.
11. An enzyme product according to any one of claims 1, 4, 6, 7, 8 and 9, characterized in that the enzyme is pig liver esterase.
12. An enzyme product according to any one of claims 1, 4, 6, 7, 8 and 9, characterized in that the enzyme is β -lactamase.

Patentansprüche

1. Stabilisiertes, biologisch aktives, wasserlösliches Enzymprodukt, gekennzeichnet durch Enzym-Moleküle mit einem zweischichtigen Schutzaufbau, der eine Polyaldehyd-Basisschicht, die mit freien Aminogruppen des Enzyms verknüpft ist, und, damit vernetzt, eine äußere Polymerschicht umfaßt, wobei die Polymere, die besagte äußere Schicht bilden, von der Art sind, daß sie im nicht-verknüpften Zustand freie Amino- und/oder Acylhydrazidgruppen umfassen.
2. Verfahren zur Herstellung eines stabilisierten, biologisch aktiven Enzymprodukts nach Anspruch 1, dadurch gekennzeichnet, daß in einem ersten Schritt ein natives Enzymprodukt in wäßriger Lösung mit einem Überschuß eines wasserlöslichen Polyaldehyds zur Reaktion gebracht wird, um ein Enzym-Zwischenprodukt herzustellen, in dem die Enzym-Moleküle mit besagter Polyaldehyd-Basisschicht überzogen sind, und in einem zweiten Schritt besagtes Enzym-Zwischenprodukt in wäßriger Lösung mit einem polymeren Reagenz zur Reaktion gebracht wird, das freie Amino- und/oder freie Acylhydrazidgruppen trägt.
3. Verfahren nach Anspruch 2, dadurch gekennzeichnet, daß das in besagtem zweiten Schritt erhaltene Produkt lyophilisiert wird.
4. Enzymprodukt nach Anspruch 1, dadurch gekennzeichnet, daß es in einer Matrix immobilisiert ist.
5. Verfahren zur Herstellung eines immobilisierten Enzymprodukts nach Anspruch 4, dadurch gekennzeichnet, daß eine wäßrige Lösung, die am Ende besagten zweiten Schrittes, wie in Anspruch 2 definiert, erhalten wird und nicht-umgesetztes Polymer enthält, einer Vernetzungsreaktion unterworfen wird.
6. Enzymprodukt nach Anspruch 1, dadurch gekennzeichnet, daß es in Form einer wäßrigen Lösung vorliegt.
7. Enzymprodukt nach Anspruch 1, dadurch gekennzeichnet, daß es ein lyophilisiertes Trockenprodukt ist.
8. Enzymprodukt nach einem der Ansprüche 1, 4, 6 und 7, dadurch gekennzeichnet, daß besagte Basisschicht aus Polyglutaraldehyd hergestellt ist.
9. Enzymprodukt nach einem der Ansprüche 1, 4, 6, 7 und 8, dadurch gekennzeichnet, daß besagte äußere Schicht aus einem Polymer hergestellt ist, das ausgewählt ist aus der Gruppe aus Polyacrylamiden, N-alkylierten Polyacrylamiden und Acylhydrazidderivaten von Polyacrylamiden.
10. Enzymprodukt nach einem der Ansprüche 1, 4, 6, 7, 8 und 9, dadurch gekennzeichnet, daß das Enzym Glukose-Oxidase ist.
11. Enzymprodukt nach einem der Ansprüche 1, 4, 6, 7, 8 und 9, dadurch gekennzeichnet, daß das Enzym Schweineleber-Esterase ist.
12. Enzymprodukt nach einem der Ansprüche 1, 4, 6, 7, 8 und 9, dadurch gekennzeichnet, daß das Enzym β -Lactamase ist.

Revendications

1. Produit enzymatique soluble dans l'eau, biologiquement actif et stabilisé, caractérisé par des molécules enzymatiques ayant une structure protectrice bicouche comprenant un revêtement de base polyaldéhyde lié à des groupes amino libres de l'enzyme, et, réticulé avec elles, un revêtement polymérique externe, les polymères constituant ledit revêtement externe étant d'un type qui a l'état non lié comprend des groupes amino et/ou hydrazide d'acyle libres.
2. Procédé de préparation d'un produit enzymatique biologiquement actif et stabilisé selon la revendication 1, caractérisé en ce que dans une première étape on fait réagir un produit enzymatique natif en solution aqueuse avec un excès d'un polyaldéhyde soluble dans l'eau pour produire un produit

enzymatique intermédiaire dans lequel les molécules de l'enzyme sont revêtues avec ledit revêtement de base de polyaldéhyde, et dans une seconde étape, on fait réagir ledit produit enzymatique intermédiaire en solution aqueuse avec un réactif polymérique portant des groupes amino libres et/ou hydrazide d'acyle libres.

5

3. Procédé selon la revendication 2, caractérisé en ce que le produit obtenu dans ladite seconde étape est lyophilisé.

10

4. Produit enzymatique selon la revendication 1, caractérisé en ce qu'on procède à une immobilisation dans une matrice.

15

5. Procédé de préparation d'un produit enzymatique immobilisé selon la revendication 4, caractérisé en ce qu'on soumet une solution aqueuse obtenue à la fin de ladite seconde étape telle que définie dans la revendication 2 et qui contient un polymère n'ayant pas réagi, à une réaction de réticulation.

20

6. Produit enzymatique selon la revendication 1, caractérisé en ce qu'il est sous la forme d'une solution aqueuse.

7. Produit enzymatique selon la revendication 1, caractérisé en ce qu'il s'agit d'un produit sec lyophilisé.

25

8. Produit enzymatique selon l'une quelconque des revendications 1, 4, 6 et 7, caractérisé en ce que le dit revêtement de base est fait de polyglutaraldéhyde.

9. Produit enzymatique selon l'une quelconque des revendications 1, 4, 6, 7 et 8, caractérisé en ce que ledit revêtement externe est fait d'un polymère choisi dans le groupe des polyacrylamides, polyacrylamides N-alcoylés et dérivés hydrazide d'acyle de polyacrylamides.

30

10. Produit enzymatique selon l'une quelconque des revendications 1, 4, 6, 7, 8 et 9, caractérisé en ce que l'enzyme est la glucose-oxydase.

11. Produit enzymatique selon l'une quelconque des revendications 1, 4, 6, 7, 8 et 9, caractérisé en ce que l'enzyme est l'estérase de foie de porc.

35

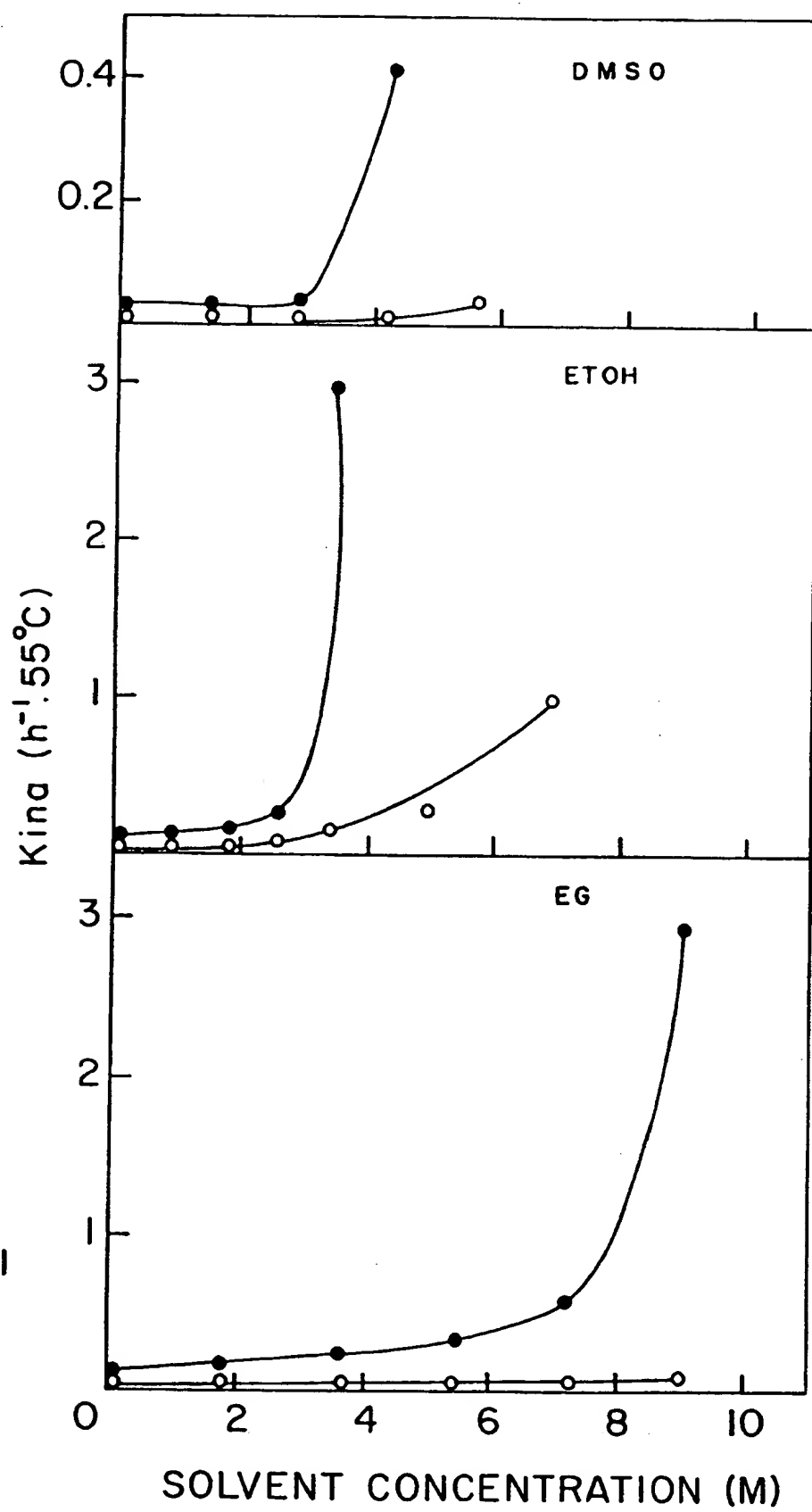
12. Produit enzymatique selon l'une quelconque des revendications 1, 4, 6, 7, 8, et 9, caractérisé en ce que l'enzyme est la β -lactamase.

40

45

50

55



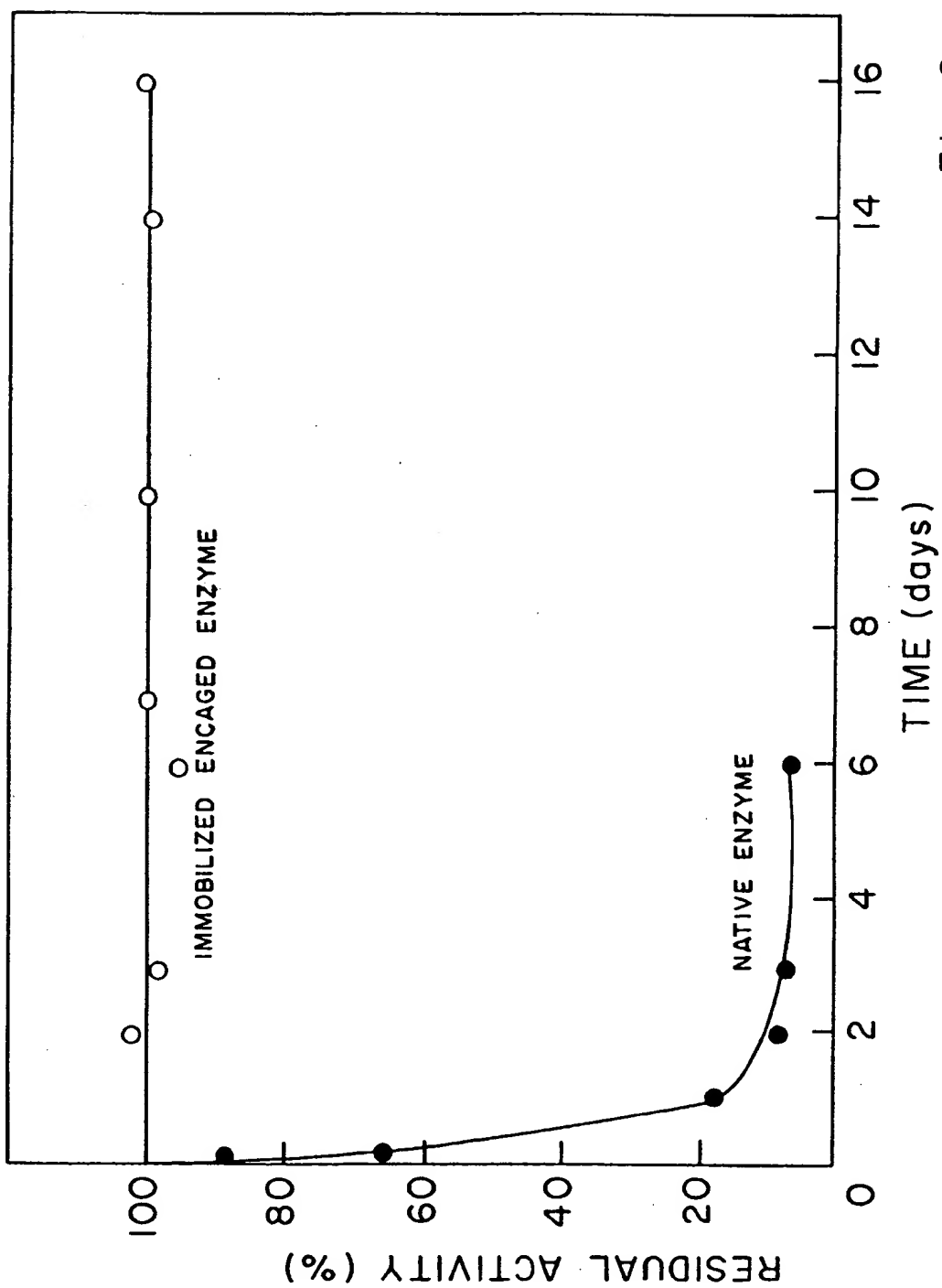


Fig. 2

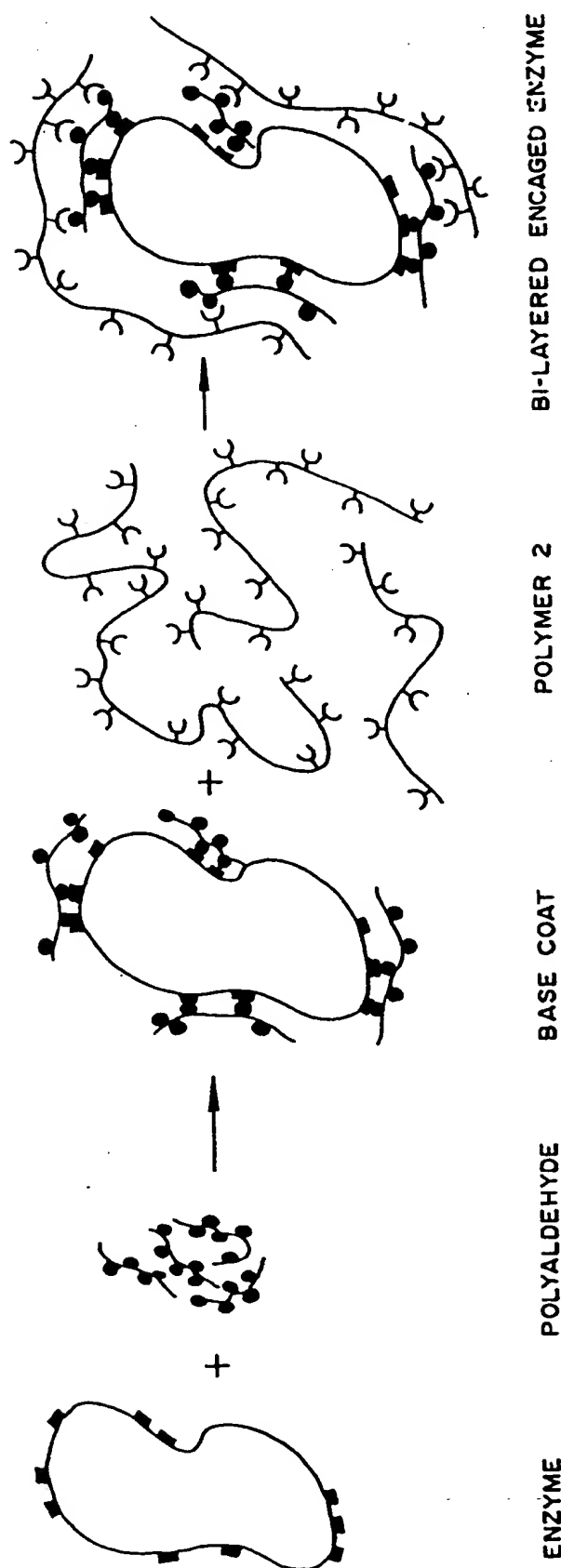


Fig. 3